

2310

INTRODUCTION

Milk is a system composed of several phases of varying degree of dispersion depending on temperature, acidity, time of standing after milking, and other factors. The continuous phase may be considered to be an aqueous solution of lactose, soluble proteins, and inorganic salts as well as various organic substances in small amounts such as urea, amino acids, lipids, and vitamins and the disperse phases as being composed of fat, the caseinate-phosphate complex, calcium phosphate, and cellular material.

The disperse phase of milk contains about three per cent of a calcium casein-phosphate complex in the form of particles, frequently referred to as micelles, varying in diameter from 40 to 300 m μ as well as approximately 3.5% fat in the form of globules with a diameter of from 0.1 to 22 μ . The larger diameter of the globules of the fat phase as well as its low solubility and density facilitates its separation from the liquid and the other components of the disperse phase. The particles of the casein complex are, however, so small that they exhibit Brownian movement. The dispersion of casein micelles in milk is remarkably stable and will withstand storage for long periods after industrial processing involving concentration, freezing, drying, and reconstitution. In fact, many of the problems of dairy technology involve the retention of the unique properties of the casein complex.

COLLOIDAL CASEINATE-PHOSPHATE PHASE

The proteins of milk occur in both the disperse and liquid phases. However, casein is the principal protein in the disperse phase, being in the form of a complex with approximately eight per cent as inorganic calcium and phosphorus together with other inorganic salts in small amounts as well as some citrate. It is reasonable to assume that the casein complex has a solubility and that there is an equilibrium between casein in the disperse phase and in the liquid

T. L. MCMEEKIN and M. L. GROVES, Pioneering Research Laboratory for Animal Proteins, Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia, Pennsylvania.

phase. This equilibrium, as well as the stability of the casein complex, is markedly affected by variations in the milk salts. Waugh⁷² has investigated the distribution of casein in micelles and in solution and concluded that the casein of milk occurs in two distinct states—in the micelles which can be removed by centrifugation and in smaller polymeric units which cannot be so removed. As much as 90% of the casein complex can be removed by ultracentrifugation. This amount can be increased by the addition of calcium chloride and reduced by diluting the milk. Waugh considered these effects to be evidence that the casein complex has a solubility and that the solubility is reduced with an increase in concentration of calcium ions.

The improved separation of the casein complex from milk by centrifugation after the addition of calcium chloride, however, must be due in part to the aggregation effect of calcium ions on the casein particles. Conversely, the greater difficulty in removing the casein complex from diluted milk is due to a disruptive effect on the micelles and is associated with a decreased particle size^{28,47} as well as a more alkaline pH, resulting in a greater net charge on the protein and an increase in repulsive forces within the micelle. As stated by Waugh,⁷³ the fact that the large casein micelles do not grow at the expense of the smaller ones is a central problem in understanding the stability of the micelles. It is possible that the soluble casein is in a different molecular form from the casein micelle and that a true equilibrium between the two forms does not exist. The increase in diffusible inorganic salts in ultrafiltrates at 3°C. as compared to 20°C. and the reversal of this temperature effect¹¹ is probably associated with the increased solubility of the casein complex at 4°C. The increased solubility of the casein complex at low temperatures is also reversed at 20°C.⁷ These effects of temperature on solubility indicate a reversible equilibrium between the soluble casein and the casein complex and are presumably associated with the greater solubility of β -casein at low temperatures.

The proteins of milk serum remain soluble in the presence of calcium salts and do not appear to have the property of forming colloidal calcium phosphate complexes as does casein. This unique property of casein may be associated with a specific arrangement of the organic phosphorus groups in casein or its unfolded structure, which could be a consequence of its large proline content,³¹ and low solubility in water.

The colloidal caseinate-phosphate particles are polydisperse. Their size distribution has been estimated by Svedberg and

Fåhræus⁶³ and Nichols *et al.*⁴⁵ by the ultracentrifuge to range from 10 to 200 $m\mu$ based on the unhydrated particle. By means of the electron microscope, Nitschmann⁴⁷ and Hostettler and Imhof²⁶ found the particles to be essentially spherical in form with diameters of 30 to 300 $m\mu$. The results for the particle sizes of the casein micelles obtained by the two methods are in general agreement when correction for hydration is applied to the former. The size distribution of the casein micelles in skimmilk are given in Table 66. It was found that the turbidity of skimmilk diluted with water decreased rapidly with time, but when milk was diluted with 0.01 molar calcium chloride or dilute formaldehyde there was essentially no change of turbidity with time, indicating that under the latter conditions particle sizes were unchanged.⁴⁷ The measurements reported in Table 66 were made with the electron microscope on milk diluted with calcium chloride and with water after formaldehyde treatment. The results were considered to be for the hydrated particles since the loss of water during drying caused the particles to become flat without a reduction in diameters. A recent electron microscope study on very thin sections of casein micelles⁵⁸ also demonstrated that the micelles are spherical, and no evidence was found for a rod-shaped particle with a length of 200 Å, as was postulated by Waugh.⁷² It was suggested that the micelle is built of spherical units of about 100 Å in diameter.

TABLE 66
SIZE DISTRIBUTION OF CASEINATE-PHOSPHATE PARTICLES IN SKIMMILK

Diameter, $m\mu^a$	Molecular Wt., Millions	Frequency, % ^b	
		<i>a</i>	<i>b</i>
40-80	10-81	20.8	32.0
80-120	81-266	35.5	34.0
120-160	266-625	23.3	23.7
160-200	625-1220	13.9	8.0
200-240	1220-2025	4.5	2.0
240-280	2025-3280	2.0	0.4

^a $m\mu = 10^{-6}$ mm.

^b *a* Diluted with 0.01M CaCl₂; *b* Fixed with formaldehyde and diluted with water derived from electron microscope photographs (after Nitschmann⁴⁷).

Choate *et al.*⁹ found that the size of a calcium-caseinate particle is a unique property of the particle and that it tends to regain the same relative order of size after dissolving and redispersing by dialysis against large volumes of milk. Thus the calcium caseinate particles of skimmilk were separated into four fractions by differential centrifugation. After the removal of the calcium by dialysis

against Veronal of pH 8.4, each of the fractions had essentially the same sedimentation value. However, after dialysis against skim-milk, the re-formed calcium-caseinate particles in each of the four fractions had unique sizes and the same relative order of size, though somewhat smaller than originally present in the four fractions, as shown in Table 67. It also appears probable that the regenerated casein particles do not have the same composition and properties of the original particles since McGann and Pyne³⁷ have found that the method used for the regeneration of colloidal casein-complex by dialysis of casein solutions against large volumes of milk does not produce a caseinate-phosphate complex with the properties of the colloidal casein-complex present in milk.

TABLE 67
SEDIMENTATION CONSTANTS FROM VARIOUS SIZED CASEINATE-PHOSPHATE PARTICLES^a

Fraction	Centrifuging Time (29,700 rpm), Min.	Sedimentation Constant of the Casein Complex S_{20w}	
		Original	Regenerated
1	1.0	980	400
2	3.8	870	280
3	10.8	640	130
4	60.0	230	116

^a After Choate *et al.*⁹

The size distribution of the casein complex has been ascribed to the amount of kappa-casein in each fraction on the assumption that the N-acetyl-neuraminic acid content is a measure of κ -casein.⁶⁰ A linear increase in the concentration of N-acetylneuraminic acid was found as progressively more casein was centrifuged from skim-milk. Therefore, its concentration was considered to be inversely correlated with the particle size distribution of casein; that is, the greater the amount of N-acetylneuraminic acid the smaller the size of the casein particle. This conclusion and the finding that the size of the casein particle is related to the colloidal phosphate content²⁷ indicates that the amount of sialic acid in a casein particle is a consequence of its surface/volume ratio as suggested by McGann and Pyne.³⁷ McGann and Pyne reached a similar conclusion based on a comparison of the nonprotein nitrogen obtained when different sized casein micelles were clotted with rennet.

Approximately three-fourths of the casein complex can be easily removed from milk by high speed centrifugation (25,000–50,000 \times g); the remaining portion is difficult to remove by this means. The undried sedimented casein complex is a relatively clear gel

which is easily dispersed by the addition of water to give an opaque colloidal suspension. The sedimented gel contains about 70% water as well as the other constituents of milk serum. De Kadt and van Minnen³⁰ have estimated the degree of hydration or the so-called "bound water" of the casein complex from a comparison of the concentration of lactose in the complex and in the milk serum. Their average value of 0.55 gm. of water per gram of protein is widely used in making corrections in concentrations of milk serum after the removal of the casein complex. Waugh and Von Hippel⁷⁴ have used a value of 0.8–1.0 gm. of water per gram of casein for hydration.

The density of the casein complex has been determined by Nichols *et al.*⁴⁵ and, more recently, by Ford *et al.*¹⁷ based on differences in density and solids (or nitrogen) in the milk before and after centrifugation. The equation of Svedberg and Chirnoaga⁶² was used for calculating specific volumes,

$$V = \frac{w - (l - h)}{\rho h},$$

where w is the weight of serum or solvent that can be contained in the pycnometer, l is the weight of solution contained by the pycnometer, h is the weight of solute in solution and ρ is the density of the serum or solvent. Values for the apparent specific volume of the dry complex for several samples of milk were found to vary from 0.692 to 0.703, corresponding to a density (l/V) of approximately 1.43. The higher value of 1.43 for the density of the casein in the complex than the value of 1.37 for casein in solution⁴⁰ is probably due to the greater density of the inorganic salts in the casein complex. By assuming that the casein complex is hydrated to the extent of 0.52 gm. water per gram of solid and that the ratio of the volume of the total particle to the weight contained in the dry complex is about 3.1 to 1, Ford calculated the apparent specific volume of the casein complex as it occurs in milk to be 0.898 with a corresponding density of 1.114. This is the density of the casein complex that should be used in calculating particle sizes from sedimentation velocities by the use of Stokes' law.

Composition of Caseinate-Phosphate Complex

The composition of the complex can be determined directly on the particles after their removal by centrifugation or ultrafiltration or indirectly by differences between the total content of the original skimmilk and its diffusate, whey or centrifuged serum. In any case,

due allowance must be made for the entrapped serum in the separated particles or the particles washed with water. A number of investigations^{18,19,30} have indicated that the casein complex is unique and acts as a single substance since it is difficult to separate it into fractions differing in composition. The early work of Van Slyke and Bosworth,⁶⁷ however, indicated that the casein particles varied in composition. They separated the caseinate complex by passing a large amount of skim milk through a separator a number of times and compared the composition of the successive deposits obtained in the separator. The first deposit contained more calcium and phosphate than the last deposit. They also removed most of the casein complex from a liter of skim milk by two successive two-hour centrifugation periods and found that the composition of the casein complex varied in the two fractions.

The results for the average compositions of the sediments obtained by Hostettler *et al.*²⁷ from five successive centrifugations of seven samples of skim milk are given in Table 68. The increase in nitrogen content of the sediment is not as great as would be expected from the decrease in phosphorus and calcium, as was noted by these investigators. This difference could be due to variations in the casein components in the sediment. Electrophoretic analyses of each of the sediments, however, indicated that the relative amount of each of the components of casein did not vary and that they were present in the same proportion as in unfractionated casein.

TABLE 68
VARIATION IN COMPOSITION OF CASEINATE-PHOSPHATE PARTICLES OBTAINED BY
SUCCESSIVE CENTRIFUGATIONS OF SKIMMILK^a
(Average of Seven Determinations)

Sediment Number	Mean Diameter of Hydrated Particles (μ)	Nitrogen, % Dry Wt.	Calcium, % Dry Wt.	Phosphorus as PO_4 , % Dry Wt.
1	0.78	12.17	3.66	6.98
2	0.184	12.45	3.19	5.94
3	0.126	13.29	3.14	5.94
4	0.084	13.17	2.93	5.80
5	0.058	13.33	2.61	4.88
% change in composition between no. 1 and no. 5		+ 9.5	-28.7	-30.1

^a Adapted from Hostettler *et al.*²⁷

The electrophoretic results reported by Heyndrickx and de Vleeschauwer²⁴ on milk serum after the removal of the larger portion of the casein complex indicate that the relative proportion of the casein components in the smaller particles differs greatly from that of the original milk. Ford and Martínez-Mateo¹⁶ isolated casein from milk serum after the removal of varying amounts of the larger casein particles by centrifugation. They emphasize that the ratio of organic phosphorus to nitrogen is the same in all fractions; however, their data show that the total nitrogen, total phosphorus, as well as organic phosphorus content of the casein particles decrease with particle size. The results of Bohren and Wenner⁷ illustrated in Table 69 also show that the phosphorus to nitrogen ratio of the small casein particles is considerably smaller than that of the total casein. The results of Annibaldi² also show that the very large particles differ in composition and properties from the smaller particles. Electrophoretic patterns of the two layers of casein, obtained by the ultracentrifugation of milk, demonstrated that the lower opaque layer contained relatively less α -casein and coagulated more slowly than did the upper transparent layer. It may be concluded from these results that there is a small change in the electrophoretic components as well as the inorganic content of casein micelles with particle size and that the smallest casein particles show the greatest difference from that of the total casein complex.

TABLE 69
P/N RATIOS OF RESIDUAL CASEIN^a

Skimmilk Centrifuged ^b	Residual Casein, % of Total Casein	P/N Ratio ^e
50,000 \times g		
180 min.—20°C.	5.7	0.045
300 min.—4°C.	15	0.044
120 min.—20°C.	15	0.054
180 min.—25°C. ^c	(99) ^d	0.055

Adapted from Bohren and Wenner.⁷
^a Casein in supernatant liquid; ^b 50,000 times gravity; ^c added Ca to the skimmilk and neutralized before centrifugation; ^d sedimentated casein; ^e P/N ratios of: whole casein = 0.055, α -casein = 0.064, β -casein = 0.04, γ -casein = 0.007.

The electrophoretic patterns obtained by the Wake and Baldwin method⁶⁹ of starch gel electrophoresis in 7*M* urea for caseinate-phosphate complex fractions obtained by centrifugation are, however, essentially the same as is shown in Fig. 19A³⁹; a, c, d represent 79% of the total casein which was obtained by centrifuging for one hour at 22,620 \times g; b, e, amounting to 14% was obtained by two hours centrifugation of the supernatant from a; the remaining

casein (f) in the supernatant was precipitated at pH 4.6, amounting to seven per cent of total casein. These electrophoretic patterns are qualitative, or semiquantitative at best, and the variations in intensity of the bands may reflect a difference in composition as has been found by chemical analyses of the fractions.⁷

The similarity of the patterns obtained by starch gel electrophoresis for the caseinate-phosphate complex (Fig. 19A) and purified acid precipitated casein preparations,⁶⁹ as shown in Fig. 19B, demonstrates that no essential fractionation occurs during the preparation of acid precipitated casein.

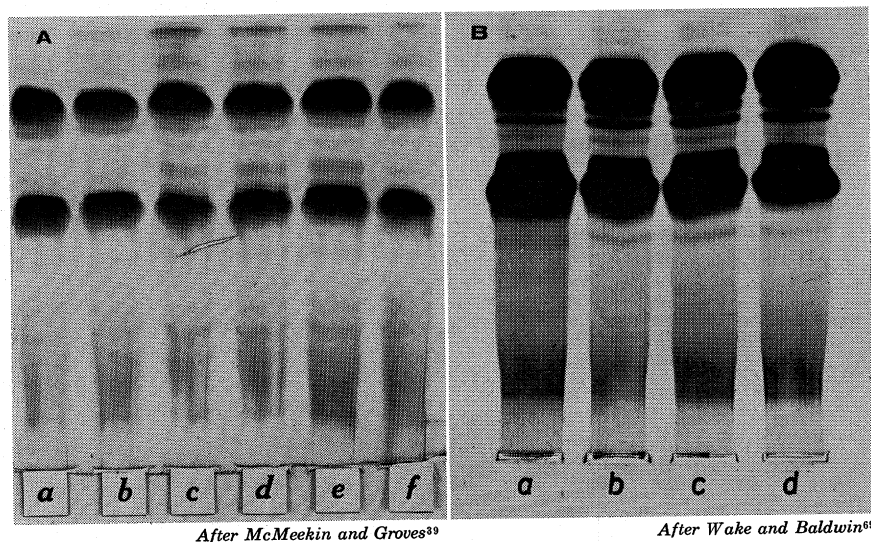
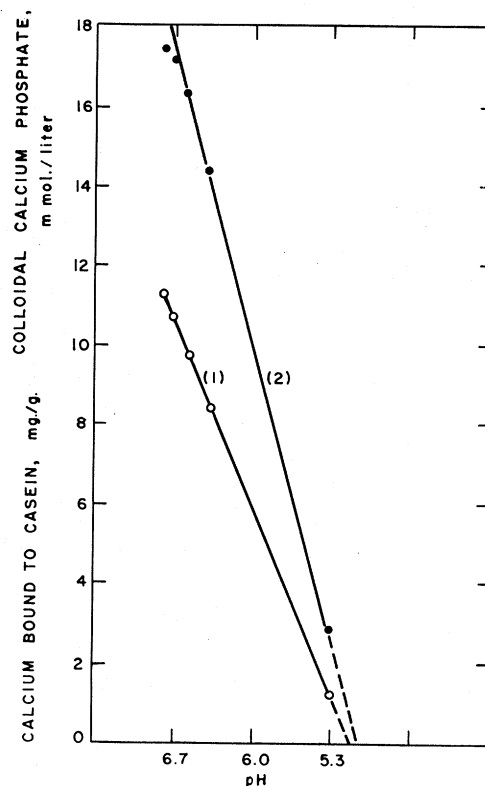


FIG. 19. CASEIN PATTERNS OBTAINED BY STARCH GEL ELECTROPHORESIS AT PH 8.6 IN 7 M UREA

A. Casein complex obtained by centrifugation at $22,620 \times g$ at 25°C . a. sediment at 1 hr.; b. sediment from supernatant (a) at 2 hrs.; c, d. sediment (a) washed with water; centrifuged, calcium phosphate removed with oxalate; e. sediment (b) washed with water; centrifuged, calcium phosphate removed with oxalate; f. acid precipitated casein pptd. from supernatant (b). B. a, b. acid precipitated casein; c. Na_2SO_4 ptd. casein; d. first cycle soluble casein.

Not only does the composition of the casein complex vary with particle size, as previously described, but wide variations in the amount of calcium and phosphorus in the caseinate-phosphate complex of milk from individual cows have also been found by White and Davies.⁷⁵ This may be due in part to small differences in the pH of the milk. Evenhuis and de Vries¹⁵ have shown that small

changes in the pH of milk produces relatively large changes in the amount of calcium bound to the casein and the amount of colloidal calcium phosphate present, as illustrated in Fig. 20. Similar results on the effect of acidity on the inorganic phosphate content of milk have been reported by Pyne and McGann.⁵¹



After Evenhuis and de Vries¹⁵

FIG. 20. THE EFFECT OF pH ON THE CALCIUM/CASEIN RATIO AND THE COLLOIDAL CALCIUM PHOSPHATE CONTENT OF MILK

(1) calcium bound to casein mg./gm. (2) colloidal calcium phosphate m. mole/liter.

Even though the caseinate-phosphate particles vary considerably in composition in different milk samples as well as in different sized casein particles,^{26,67,75} the approximate composition of the casein particles removed by centrifugation is of interest. The values for the composition of the casein particles as given in Table 70 were

derived from analysis of a broad range of particle sizes representing roughly three-fourths of the total casein complex. Values for the composition of the caseinate complex in Table 70 differ from those given in Table 68 (p. 379), since the preparations used in obtaining the data in Table 70 were washed free from the constituents of milk serum, while the preparations used in obtaining the data in Table 68 were not washed.

TABLE 70
COMPOSITION OF CALCIUM CASEINATE-PHOSPHATE PARTICLES OBTAINED FROM SKIMMILK BY CENTRIFUGATION

Constituent	Caseinate-Phosphate, gm./100 gm. (Washed and Dried)
Casein (N x 6.4)	93.4 ⁵⁴ 93.1 ¹⁸
Calcium	2.98 ⁵⁴ 2.75 ¹⁸ 2.89 ⁶⁷
Magnesium	0.11 ¹
Sodium ^a	0.11 ¹¹
Potassium ^a	0.26 ¹¹
Organic phosphorus (PO ₄)	2.26 ⁵⁴
(Casein)	2.36 ¹⁸
Inorganic phosphorus (PO ₄)	2.94 ⁵⁴ 2.84 ¹⁸
Citrate (as citric acid) ^a	0.4 ¹²

^a Obtained by difference between the amount present in milk and in the aqueous phase.

The constancy of the casein composition, as shown by electrophoretic analyses, of successive fractions of the complex obtained by ultracentrifugation^{7,30} indicates that the casein components of the complex strongly interact in a manner similar to the components in acid precipitated casein. Sullivan *et al.*⁶¹ found that in skim milk at low temperatures some of the casein originally present in micellar form is converted to nonmicellar casein and that a partial fractionation of α - and β -casein could be accomplished by centrifuging skim milk at low temperatures. Warner⁷⁰ had previously shown that β -casein could be separated from α -casein at low temperatures from solutions of acid casein because of its greater solubility. These results indicate that properties of the purified individual components of the casein complex are reflected in the properties of the casein micelles. Sullivan *et al.*⁶¹ found that β -casein had a sedimentation constant of 1.57 below 15°C. and forms aggregates at room temperature. The sedimentation value of α -casein at 8°C. was found to be $S_{20} = 3.99$. From sedimentation and diffusion constants, molecular weights of 24,000 and 121,800 were calculated respectively

for β - and α -casein. They concluded that since complete disaggregation of α -casein was not observed under environmental conditions resembling those found in milk that the α -casein would be present in some form of aggregates in milk.

Structure of Caseinate-Phosphate Complex

The fact that casein in milk is always associated with calcium phosphate and that it cannot be separated from the calcium phosphate by centrifugation indicates chemical combination between casein and calcium phosphate since the density of calcium phosphate is more than twice that of casein. Much work and speculation have been devoted to the nature of the association of calcium phosphate with casein. Is it a mixture of colloidal calcium phosphate with colloidal calcium caseinate or does it have a structure such as a double salt? Van Slyke and Bosworth⁶⁷ interpret their preparative and analytical results on the casein complex as indicating that the casein and inorganic calcium phosphate are not combined. Their opinion is based on the wide variations in the ratio of casein phosphorus to inorganic phosphorus in the centrifuged deposits which they found to vary from 1:1.2 to 1:1.9. They concluded from their results that the calcium and phosphorus remaining after calculating the amount of calcium associated with the organic phosphorus of casein was in the ratio of dicalcium phosphate. If it is assumed that casein contains 0.85% organic phosphorus instead of 0.71%, as they did, and that an equivalent amount of calcium is associated with the organic phosphorus of casein, their analytical results for the remaining calcium and phosphorus are in better agreement with the ratio of tricalcium phosphate though the variations in individual milk samples are large. Ramsdell and Whittier⁵⁴ determined the composition of the washed caseinate-phosphate complex obtained by centrifugation. They found that inorganic phosphorus and calcium remaining, after assuming that 1.18% of the calcium was combined with the casein, was present in the ratio of tricalcium phosphate. Titration results obtained after treating the casein complex with potassium oxalate were also consistent with the presence of tricalcium phosphate. De Kadt and van Minnen³⁰ and others^{18,53,54} have calculated from data on the caseinate-phosphate sediments obtained by centrifugation that after assigning calcium equivalent to the organic phosphorus of casein, the ratio of the remainder of the calcium to the inorganic phosphorus is that of tricalcium phosphate, namely 1.5. This result, as well as the data obtained from the titration of milk or

the isolated caseinate-phosphate after the addition of potassium oxalate,⁵² is considered to be evidence for the presence of tricalcium phosphate in the caseinate particles. It has been assumed that the tricalcium phosphate forms a complex ion with calcium which is bound to organic phosphate of casein as follows:³⁰ $\text{casein}-\text{PO}_4 = \text{Ca} \dots \text{Ca}_3(\text{PO}_4)_2$. While this type of structure is consistent with the calculation of the ratio of the calcium phosphorus of 1.5, it assumes that the amount of calcium bound to casein is constant, whereas White and Davies⁷⁵ report that the amount of calcium in calcium caseinate varies from 9 to 16 mg/gm. casein. It might also be expected that if a complex of caseinate-phosphate is formed, the ratio of the casein phosphorus to inorganic phosphorus would be constant. As previously indicated, Van Slyke and Bosworth found wide variations in the ratio of casein phosphorus to total phosphorus. More recent analyses⁷⁵ also indicate that the amount of inorganic phosphorus associated with the caseinate-phosphate complex varies from 11 to 25 mg/gm. casein in samples of milk from individual cows. The amount of calcium and phosphorus also decreases with successive sediments from mixed milk, as is shown in the results in Table 68. These findings constitute strong evidence that the composition of the casein complex varies widely even though the results on mixed milk indicate uniformity of calcium to phosphorus ratios.

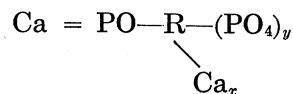
The assumptions involved in calculating the ratio of the calcium:phosphate as well as the amount of calcium bound to casein are of questionable validity. There is general agreement that isolated casein from mixed milk contains about 0.85% phosphorus; however, no data are available on the phosphorus content of casein from the milk of individual cows. The usual method of calculating casein phosphorus is to subtract the acid soluble phosphorus from the total phosphorus. This involves the errors contained in two analytical determinations as well as an assumption that the difference between these two phosphorus values represents the casein phosphorus. The finding of Graham and Kay²¹ that the acid insoluble phosphorus includes as much as 25% of lipid phosphorus illustrates the magnitude of error involved in attributing this phosphorus value to casein.

Evenhuis and De Vries¹³ have investigated the calcium plus magnesium phosphate precipitated on yeast cells during the heating of skimmilk at 120°C. They conclude that it has the composition of apatite with a ratio of $(\text{Ca} + \text{Mg})/\text{PO}_4$ of 1.67. They¹⁴ also found that the precipitate obtained by neutralizing acid whey

contained 50% calcium phosphate, 25% protein, and the remainder unaccounted for. The acid extractable portion of this precipitate contained the $(\text{Ca} + \text{Mg})/\text{PO}_4$ ratio of 1.6. They assume a value of 1.6 for the ratio of $(\text{Ca} + \text{Mg})/\text{PO}_4$ for the tricalcium salt in the casein complex. The mole ratio of Ca/Mg in the casein complex is about 15/1.¹ Evenhuis and De Vries conclude that the calcium phosphate as such is present in all milk in a finely divided state and that the amount of calcium bound to the casein varies with the composition of the milk. They postulate that, at temperatures above 70°C., calcium phosphate with a ratio of 1.67 recrystallizes and the velocity of crystallization increases with temperature. After cooling, heated milks are considered to be somewhat unsaturated with calcium and magnesium, which explains the stability of heated milks.

No mechanism for the precipitation of tricalcium phosphate by yeast cells during the heating of milk has been suggested though it is known that the yeast must be present during the heating in order to precipitate calcium phosphate.⁸ The yeast cells are treated with acid prior to their use in precipitations of calcium phosphate from heated milk. It appears possible that the acid-treated yeast may act as an ion exchange material in competing with casein for the calcium and phosphate.

Van Der Burg⁸ also favors the idea of a chemical bond between the casein and inorganic phosphate and calcium. He considers casein to be a large ion with a number of positive and negative bonds scattered over its surface depending on the pH of the solution. He suggests a general formula for the caseinate complex as follows:



This formula accounts for the variable amounts of calcium and phosphate associated with the caseinate complex and has merit in being consistent with many of the properties of the caseinate, such as the finding of Eilers¹² that preparations of the caseinate-phosphate usually did not show the grid for calcium phosphate in the Debye-Scherrer diagram. In view of the heterogeneity of the caseinate, as shown by the calcium and phosphate contents of successive sediments (Table 68), and the great number of its protein components, as shown by gel electrophoresis (Fig. 19A, p.381), it is obvious that no formula of constant composition for the structure of the caseinate-phosphate is justifiable.

The theory that the binding of calcium and phosphate to casein is essentially ionic has been expanded by Ter Horst.⁶⁵ She considers that phosphate ions combine with cationic ions of the casein, such as the epsilon amino group of lysine, and that calcium combines with anionic groups, such as carboxyl groups and the phosphoric acid ester group of casein. This hypothesis is consistent with the variable inorganic content of the casein complex, and it is reasonable that a portion of the calcium should be bound in this manner. However, the fact that casein is the only milk protein that forms a colloidal complex with calcium phosphate indicates unique structural requirements. The experiments of McGann and Pyne³⁷ demonstrated that the casein complex of milk is destroyed by acidification at low temperature and that it does not re-form when acidified milk is dialyzed against large volumes of milk where there is ample opportunity to recombine with calcium and phosphate ions. Reeves and Latour⁵⁵ found that the phosphopeptides prepared from casein have the property of sequestering calcium phosphate, which suggests that these groups have similar properties in the intact casein molecule.

Manson⁴² has obtained evidence for two kinds of calcium linkages in the casein complex. When the calcium casein complex, prepared by centrifuging skim milk, is treated with a limited amount of potassium oxalate, the complex dissolves but gives only one component by gradient density electrophoresis.⁴³ When the soluble complex is treated with an excess of oxalate ion or made acid, two electrophoretic components with mobilities of α - and β -casein are formed. These results are considered to be evidence that the first oxalate added breaks calcium bonds concerned with forming micelles and that the further addition of oxalate removes calcium that binds the components of casein together.

Based on viscosity changes in concentrated skim milk produced by urea, alkali and complexing agents, Beeby and Kumet⁵ consider that in the casein micelle there is an equilibrium between the disruptive and stabilizing forces. The micelle is thought to expand before disruption and that the expanded volume causes an increase in viscosity. Formaldehyde and also calcium ions stabilize the casein micelles. Raising the pH increases the repulsive forces within micelles causing disruption. Calcium, either in the ion atmosphere or as bridges, as well as hydrogen bonds are considered to be the two major factors in maintaining the stable structure of the casein micelle.

Properties of the Caseinate-Phosphate

The remarkable stability of the casein particles in milk and the changes produced during its processing—involving heating, freezing, evaporation, storage, and reconstitution—has been the subject of numerous investigations. White and Davies⁷⁵ have compared the stability of the casein complex with the composition of milk from individual cows and as a function of the lactating cycle. In the mid-lactating period the composition was similar to that of herd milk, while in the late lactation period the concentration of colloidal salts was high and the soluble portion low. Precipitation of the casein complex by 60 to 90% alcohol was related to the ionized calcium content. The rennet coagulation time of individual milks varied from 1.4 to 12 min. and two samples did not coagulate, presumably due to a high pH. Wide variations in the heat stability of samples of milk were found; however, no correlation was found with the composition. This investigation illustrates the difficulty of isolating specific factors involved in the stability of the casein complex in milk.

Rose⁵⁷ found that the stability of milk from individual cows to temperatures of 140°C. varied considerably during the lactating cycle. Small changes in pH of the milk were an important factor in its stability. Stability was at a maximum between pH 6.6 to 6.7 and a minimum between pH 6.7 to 6.9. Milk with a maximum heat stability also gave some correlation with the ratio of the soluble calcium to the inorganic phosphorus though the degree of correlation was not high.

Methods have been developed for investigating the properties of the caseinate-phosphate complex other than direct observations on milk or on the casein complex after separation by centrifugation. Thus the role of colloidal phosphate in milk has been evaluated by McGann and Pyne³⁷ who have prepared milks of the following kind: (1) milk with decreased colloidal phosphate by acidification followed by dialysis against large volumes of milk; (2) milk with an increased colloidal phosphate content by adding sodium hydroxide to milk and dialyzing against the original milk; (3) a low soluble phosphate milk by dialysis against a synthetic mixture of salts and lactose free from soluble phosphate; (4) a synthetic milk prepared from colloidal phosphate-free milk by the addition of calcium chloride, sodium phosphate, citrate, and hydroxide required to re-form the original content of colloidal phosphate; and (5) a small particle milk by removing the larger casein particles by centrifugation. Colloidal phosphate-free milk is translucent as compared

to ordinary milk. The progressive removal of colloidal phosphate causes a steady increase in the viscosity until it is about 30% greater than milk. It is believed that the increase in viscosity caused by the removal of the colloidal phosphate is due to a change in the size and shape of the casein micelle. Normal milk is remarkably stable to calcium ions, as shown by the fact that milk can be made to one molar with calcium chloride without precipitation, providing the pH is not subsequently adjusted. In colloidal phosphate-free milk, on the other hand, the casein is precipitated by only 25 micromoles of calcium chloride per liter. These results indicate that the colloidal phosphate of milk is concerned with its stability and may serve to preserve micelle formation.

In the separation of the κ -casein fraction, as described by Waugh and Von Hippel,⁷⁴ it is essential that the natural micelles of milk be destroyed by oxalate, which effectively removes the colloidal phosphate and calcium from the caseinate, before fractionation with 0.25M CaCl₂. Normal milk does not give a precipitate with calcium chloride at 0° or 37°C.,³⁷ whereas the colloidal phosphate-free milk does. These results suggest that the presence of colloidal-caseinate phosphate prevents the separation of the κ -casein fraction from the other casein components and maintains the integrity of the micelles. Colloid phosphate-free milk coagulates with ethanol in the same manner as does normal milk; however, the colloidal phosphate-free milk is somewhat more resistant to heat coagulation than normal milk. Small particle milk has approximately the same properties as normal milk diluted to the same casein content even though small particle casein has been found to contain more sialic acid or κ -casein.⁶⁰ The results obtained by Pyne and McGann⁵¹ on the titration of milk and colloidal phosphate-free milk with formaldehyde indicate that the presence of colloidal phosphate restricts the accessibility of the casein to formaldehyde. They also found that citrate is firmly bound to the caseinate-phosphate complex and that its presence favors the formation of tricalcium phosphate. These effects of colloidal phosphate on the properties of calcium caseinate suggests some type of chemical union between the colloidal phosphate and the calcium caseinate.

Factors involved in the stability of the casein complex have also been revealed by investigations on the properties of the casein components obtained by chemical fractionation. Thus, Linderstrøm-Lang³⁵ in his classical studies on the heterogeneity of casein found that the casein fractions varied in phosphorus content,

precipitability with calcium ions, and time of clotting with rennin. Based on these differences in properties of the casein components, Linderstrøm-Lang considered that casein is a colloidal mixture of proteins and that rennin causes the coagulation or clotting of casein by splitting a minor component which is concerned with stabilizing the colloidal system. More recently, Waugh and Von Hippel^{68,74} have fractionated casein and studied the interaction of the fractions in the presence of calcium ions. They separated the casein from milk in the form of micelles by the addition of calcium chloride to skim milk to make a concentration of 0.12*M* followed by centrifugation for 90 min. at $45,000 \times g$ (see Chapter 3). Calcium was removed and the micelles destroyed by the addition of potassium oxalate and oxalic acid at 0°C. After the removal of calcium oxalate by centrifugation and the excess of potassium oxalate by dialysis, the casein was fractionated by adding calcium chloride to the casein solution, maintained at pH 6.7–7.0, to give a final concentration of 0.25*M* calcium chloride. The casein fraction insoluble in calcium chloride was removed by centrifugation at 37°C. and found to be a mixture of α - and β -casein. The fraction soluble in calcium chloride contained β -casein and a factor responsible for the stabilization of micelles, which they designated κ -casein. It was estimated from electrophoretic studies that casein contained about 15% κ -casein. When calcium ions were added to a solution of the casein fraction, insoluble in calcium chloride, a flocculent precipitate was formed; however, if the soluble fraction (containing κ -casein) was added to the calcium insoluble casein fraction before adding calcium ions, a stable suspension of calcium caseinate particles was obtained. Further purification of the casein components and the conditions for their interaction have been described by Waugh.^{72,73} The calcium caseinate micelles are considered to be composed of α_s - and β -, κ -, M-caseins (see chapter 3). At 25°C., α_s - and β -casein are insoluble in calcium chloride while κ - and M-casein are soluble. Under appropriate conditions, all of these caseins interact; however, the most important interaction is believed to involve three molecules of α_s -casein and one molecule of κ -casein to form a complex which is stable and is not precipitated by 0.03*M* calcium chloride at 25°–37°C. but is unstable at 0°C., being dissociated by calcium chloride.

These findings of Linderstrøm-Lang's and of Waugh and Von Hippel's concerning the interaction of casein components to form colloidal calcium caseinate in the presence of calcium ions are of great interest and obvious importance in understanding the forces

involved in the calcium-caseinate micelles of milk; however, the direct application of these results obtained on simple artificial systems to the properties of the calcium-caseinate micelles of milk requires considerable extension of our knowledge. Thus, the calcium-caseinate micelles of milk contain approximately eight per cent of inorganic calcium phosphate absent in the artificial systems. Pyne and McGann's^{37,51} experiments have indicated the great effect of the inorganic calcium phosphate on the stability of the calcium-caseinate micelle to calcium. A further difference in the two systems is the fact that κ -casein does not interact with α_s -casein in a weight ratio of 1 to 4 at 0°C. to prevent the precipitation of α_s -casein by calcium ions while in milk the caseinate-complex is not precipitated by calcium chloride between 0° and 37°C.

Size of the Caseinate-Phosphate Phase and Its Protein Components

The approximate molecular weight of the caseinate-phosphate particles has been calculated by Nitschmann⁴⁷ from electron microscope measurements and was found to vary from 10 to 3,280 million. Similar results have been obtained from measurements made with the ultracentrifuge. Much work has also been done on the size of isolated casein and its components. Pedersen⁴⁸ has determined the rate of sedimentation of the proteins in skim milk after dialysis against phosphate buffers, which converts the insoluble caseinates into soluble salts. In the sedimentation diagram (Fig. 21) the peaks designated with Greek letters indicate protein components. The α -component may be ascribed to α -lactalbumin which has been isolated by Gordon and Semmett.²⁰ The β -component was found to be identical with β -lactoglobulin and the γ -component was considered to be the immune globulin of milk serum. The remaining six peaks were found to be associated with casein. This sedimentation diagram indicates the heterogeneity of casein. Most of the casein, however, has a sedimentation constant of $S_{20} = 10.4$ (Fig. 21, δ). Much experimentation has been done on the separation of the casein components (see Chapter 3); however, aggregation or polymerization, as well as complex formation, is difficult to evaluate or overcome. Numerous values for the sedimentation constants and molecular weights for casein and casein components have been reported though the interpretation of some of these values are of transitory significance because of the heterogeneity of the casein preparations and also because of the inadequacy of the method used for determining size.

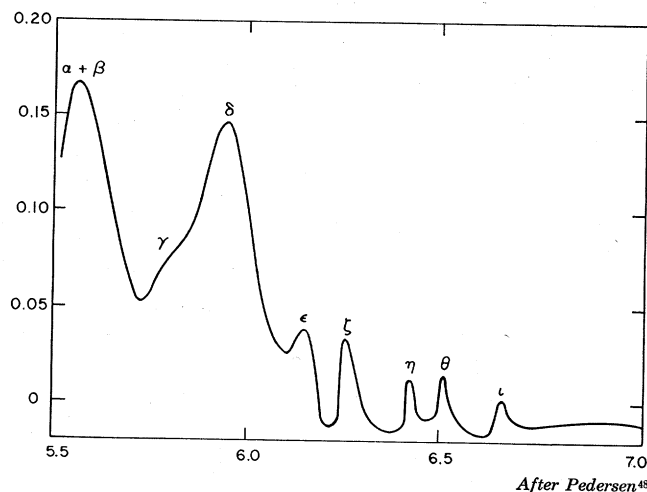


FIG. 21. ULTRACENTRIFUGAL SEDIMENTATION DIAGRAM FROM DIALYZED SKIMMILK OBTAINED BY THE REFRACTION METHOD

The abscissa (x in cm) represents distances from the center of rotation and the ordinates (z in mm.) represent the scale line displacements which are proportional to the concentration gradient. Each peak on the diagram corresponds to the sedimentation of molecules of a certain size.

Sullivan *et al.*⁶¹ discovered that the sedimentation constant of β -casein is temperature dependent. At room temperature the major component sediments rapidly while the minor component settles slowly. When the temperature is lowered below 15°C. the fast sedimenting component disappears indicating that at room temperature there is an equilibrium between aggregates and a monomer which shifts to the monomer form below 15°C. α -Casein shows only one component at room temperature and 8°C. Although Von Hippel and Waugh⁶⁸ used unfractionated casein, their results for the sedimentation constant of the β -casein component gave values in agreement with those reported by Sullivan *et al.*⁶¹ The sedimentation constants and molecular weights of a number of purified casein components are summarized in Table 71. It may be noted that with the exception of the molecular weight reported by Sullivan *et al.* for α -casein, determined at pH 7.8, the values reported for the molecular weights of the casein components are relatively small. The low values for the molecular weights of α - and κ -casein are in part a consequence of making measurements on solutions of pH values of 11–12 following Waugh and Von

Hippel's^{68,74} finding that the values for the sedimentation constants are much lower at these high pH values. It has been suggested that these low values for the molecular weight at pH 11–12 may be due to the splitting of the disulfide bonds.⁶⁴ This is particularly true of κ -casein which gave a sedimentation constant of 13.4 at pH 8.5 and a value of 1.4 at pH 11–12. The large value of the sedimentation constant at the lower pH is considered to be due to aggregation by Waugh and Von Hippel. It appears likely that at the pH of milk (6.7) the protein components are aggregated since in the case of β -casein the effect of temperature on the aggregation appears to be the same on the β -casein in milk as on the isolated β -casein.

TABLE 71
SEDIMENTATION CONSTANTS AND MOLECULAR WEIGHTS OF CASEIN COMPONENTS

Component	pH	$S_{20} \times 10^{13}$	Molecular Weight
α_1 -Casein ⁶¹	7.8	4.0	121,800
α_1 -Casein ⁸⁸ (free of κ -casein)	11.0	1.8	24,800
α_1 -Casein ⁸⁸ (free of κ -casein)	6.8	4.4	...
α_1 -Casein ⁸⁸ (free of κ -casein)	7.3 (6.6 <i>M</i> urea)	...	27,600
α_1 -Casein ⁴⁶	4.8 ^a	...	27,800
α_2 -Casein ⁷²	11–12	1.6	23,300
α_1 -Casein ⁴¹	7.1	3.1	...
α_3 -Casein ²⁵	7.0	12–23	...
β -Casein ⁶¹	7.8	1.6	24,100
β -Casein ⁸⁸	11	1.2	17,300
β -Casein ⁸⁸	7.0 (6.6 <i>M</i> urea)	...	19,800
β -Casein ⁴⁶	4.8 ^a	...	23,100
κ -Casein ⁶⁴	... (7.0 <i>M</i> urea)	...	60,000
κ -Casein ⁷²	6.9	13.4	...
κ -Casein ⁴	50,000
κ -Casein ⁷²	11–12	1.4	16,300
κ -Casein ⁸⁸	12	...	26,000
κ -Casein ⁸⁶	7.0	13.1	...
λ -Casein ³⁶	7.0	1.1	...

^a By osmotic pressure in 6.6*M* urea.

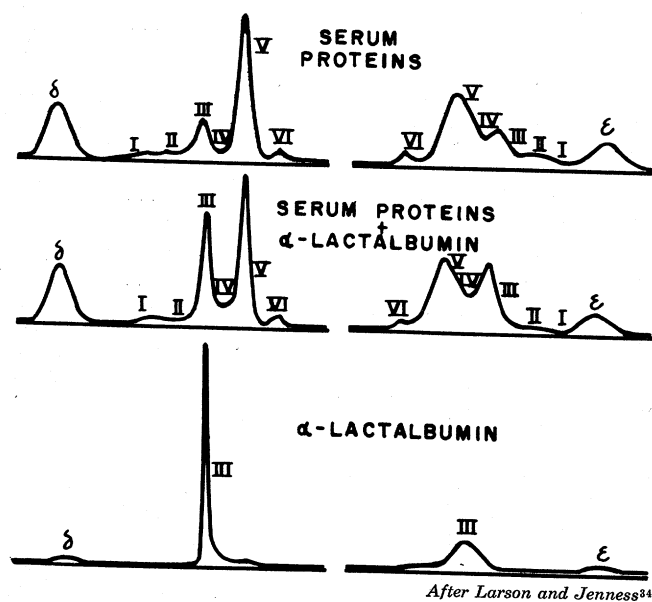
The interaction of casein components to form complexes has been demonstrated by Waugh⁷² who found that by mixing α_s -casein with κ -casein that α_s - κ -casein complex re-formed immediately giving characteristic ultracentrifuge patterns showing that α_s - κ -casein complexes are stoichiometric interaction products involving approximately 4 parts dry weight of α_s -casein and 1 part of κ -casein. Waugh considers the casein components to have the shape of cylinders and has calculated frictional ratios from sedimentation

data. He suggests that the polypeptide chains of the casein components exist in a coiled form and that each can be represented by a cylinder 16 Å in diameter and a length proportional to the molecular weight. Thus, the length of α_s -casein was calculated to be 210 Å; β -casein 215 Å; and κ -casein 150 Å. The previously cited electron microscope measurements of Shimmin and Hill⁵⁸ are inconsistent with these dimensions.

THE LIQUID PHASE

Milk Serum Proteins

Milk serum contains about 0.6% protein of which β -lactoglobulin and α -lactalbumin predominate together with numerous minor proteins and enzymes (see Chapter 3). In common with most proteins, milk serum proteins combine with calcium;⁷⁷ however, their calcium salts do not form colloidal solutions, as does casein; consequently, they are found in the aqueous phase of milk. The electrophoretic composition of the serum proteins as determined by Larson and Jenness³⁴ using the Tiselius method is shown in Fig. 22. The protein components (peaks) are numbered in accordance with

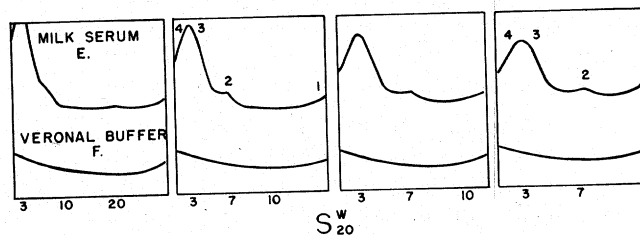


After Larson and Jenness³⁴

FIG. 22. ELECTROPHORETIC COMPARISON OF THE MILK SERUM PROTEINS; MILK SERUM PROTEINS WITH ADDED α -LACTALBUMIN; AND α -LACTALBUMIN, pH 8.6, 0.1 IONIC STRENGTH

their increasing mobilities. The component with the greatest mobility (VI) is considered to be the same as the albumin of blood serum. β -Lactoglobulin (V) is the most abundant protein in milk serum. This figure is of particular interest in that it illustrates a good method for identifying a protein peak in an electrophoretic pattern of a mixture of proteins providing the pure protein is available. The top electrophoretic pattern is that of milk serum. The middle pattern shows the effect of adding pure α -lactalbumin to milk serum on its electrophoretic pattern. The lower electrophoretic pattern is that of pure α -lactalbumin. A comparison of these electrophoretic patterns demonstrates that peak No. III corresponds to α -lactalbumin. Components I and II have been found to be associated with the immune lactoglobulin of milk.

The size distribution of the proteins in milk serum are indicated by the ultracentrifuge patterns obtained by Larson *et al.*³³ in Fig. 23 as well as that of the ultracentrifuge pattern of milk previously de-



After Larson *et al.*³³

FIG. 23. ULTRACENTRIFUGAL SEDIMENTATION DIAGRAM OF MILK SERUM PROTEINS AT 24, 40, 56, AND 72 MIN. AFTER ROTOR HAD ATTAINED THE SPEED OF 59,780 R.P.M. ANALYSES WERE MADE AT 24° IN VERONAL BUFFER OF PH 8.6 AND 0.1 IONIC STRENGTH

scribed in Fig. 21 (p. 392). Since the sedimentation properties for α -lactalbumin and β -lactoglobulin are similar, the two proteins are not resolved in separate peaks; however, Component No. 4 in Fig. 23 was identified as α -lactalbumin with a sedimentation constant of $S_{20W} = 1.8-2.0$; No. 3 as β -lactoglobulin $S_{20W} = 2.7-3.2$; Components No. 1 and 2 as the immune globulin with a $S_{20W} = 19.9$ and 6.8, respectively. Serum albumin, identical with blood serum albumin, apparently is not present in a sufficient concentration to be seen in these patterns.

A number of the enzymes and minor proteins of milk serum are also found associated with the fat phase of milk, probably being absorbed at the fat/serum interphase. Hetrick and Tracy²³ found

that 40% of the alkaline phosphatase of milk is in the cream while 60% is associated with the skim milk and that when casein is precipitated, about half of the phosphatase remaining in the skim milk is removed with the casein. An increase of separation temperatures up to 49°C. did not appreciably change the distribution of the phosphatase in the cream and skim milk fractions. Morton⁴⁴ also found that milk microsomes (lipoprotein particles) associated with the cream contained about one-half of the total milk phosphatase. However, the large caseinate particles obtained by centrifuging skim milk were reported to be less than six per cent of the total phosphatase and no xanthine oxidase, cytochrome oxidase, or dehydrogenase. Xanthine oxidase was found to be equally distributed between milk microsomes and skim milk. Zittle *et al.*⁷⁶ found much more xanthine oxidase and alkaline phosphatase in cream than in skim milk. These enzymes were found to be associated with the lipoprotein fraction in both skim milk and cream and could be separated by high speed centrifugation. According to Polis and Shmukler,⁴⁹ the concentration of aldolase is twice as great in cream as in skim milk.

The proteolytic enzyme of milk is precipitated with casein at pH 4.7.⁷¹ In view of the finding of Groves²² that the red protein of milk, as well as phosphatases and the proteolytic enzyme associated with the casein fraction, could be removed from casein by acid extraction, it appears likely that enzymes are absorbed from whey when casein is precipitated at pH 4.7 and that they probably are not a component of the caseinate-phosphate complex of milk. It may be inferred from these studies on the distribution of enzymes that the enzymes associated with lipoproteins are in equilibrium between the cream and the aqueous phase but are concentrated in the cream. The remainder of the enzymes of milk are largely in the aqueous phase but some are adsorbed on the casein when milk is acidified.

Salt Content of Milk Serum

The salt content of milk serum separated by several methods has been determined by Davies and White.¹¹ Their average results for two milks, obtained by separating milk serum by diffusion, centrifugation, and clotting with rennet, are compared in Table 72. These values are in essential agreement with each other and the very extensive previous results on the composition of the nonprotein aqueous phase of milk by the same authors.⁷⁵

TABLE 72
DISTRIBUTION OF SALTS (IN MG./100 GM. MILK) BETWEEN DISSOLVED AND COLLOIDAL
STATE IN MILK^a

	Dissolved				Colloidal State
	Total in Milk	Diffusate (20°C.)	Whey	Centrifuged Serum	(Average 3 Methods)
Total calcium	114.2	38.1	39.9	40.9	74.6
Ionized calcium	...	11.7	11.6	11.9	...
Magnesium	11.0	7.4	7.8	8.1	3.3
Sodium	50	46	47	47	3.3
Potassium	148	137	143	141	8.0
Total phosphorus	84.8	37.7	37.4	37.9	47.1
Inorganic phosphorus	...	31.8	30.8	31.8	...
Citric acid	166	156	152	154	12.0
Chloride	106	106.5	106.2	105.6	...
Total nitrogen	...	20.7	124.6	110.7	...
Casein nitrogen	364	0	21.6	6.8	...
Lactose ^b	4800	4800	4800	4800	...

^a Adapted from Davies and White¹¹

^b Average of two separated milks, corrected for bound water.

Equilibria Among Salts and Ions in Solution

The principal dissolved salt constituents of milk consist of phosphates, chlorides, sulfates, and bicarbonates of calcium, magnesium, sodium, and potassium. The determination of the ions present and their concentrations is an intricate problem. The principles involved in the equilibria salts of milk and their effect on the other constituents of milk have been extensively reviewed by Pyne.⁵⁰ The concentrations of ions in milk from individual cows vary widely, as is illustrated by the extensive data of White and Davies.⁷⁵ With the exception of chlorides, and probably sulfates also, the most abundant ions of milk are present in both the colloidal phase and dissolved phase, as given in the data in Table 72. The manner in which the calcium, magnesium, phosphate, and citrate are associated with the casein in the complex is difficult to evaluate though it seems likely that calcium, magnesium, sodium, and potassium are combined with the anionic groups of casein in an amount equivalent to the base-combining capacity of casein at the pH of milk, namely, 6.7.

The salts of the strong acids can be considered to be completely dissociated, whereas the salts of the weak acids—namely, carbonic,

citric, and phosphoric—present in milk, are distributed in accordance with the Henderson-Hasselbach equation:

$$\text{pH} = \text{pK}_a + \log \frac{[\text{Salt}]}{[\text{Acid}]}$$

From the dissociation constants of the acids (pK_a's) the ratio of the ionic forms can be calculated. Their dissociation constants are as follows:

Acid	pK ₁	pK ₂	pK ₃
Citric ⁵⁶	3.13	4.76	6.40
Phosphoric ⁶	2.23	7.21	12.32
Carbonic ⁵⁶	6.35	10.33	

Since the total ionic concentration influences dissociation constants, the values given are only an approximation of those to be found in milk. In Fig. 24 the distribution of the ionic forms of phosphoric, citric, and carbonic acids as a function of pH is shown. At the pH of milk, the mono- and diphosphate, di- and tricitrate, and bicar-

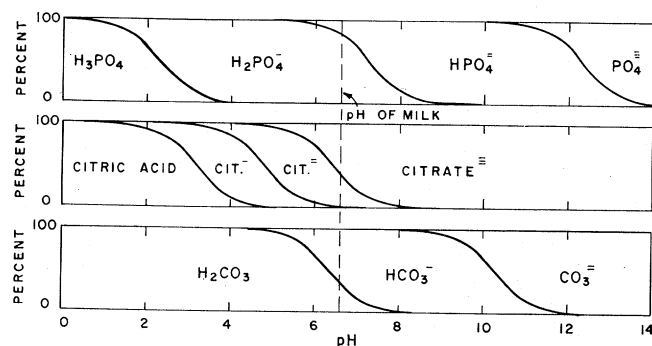


FIG. 24. IONIZATION OF THE WEAK ACIDS FOUND IN MILK

bonate and carbonic acid ($\text{CO}_2 + \text{H}_2\text{O}$) forms predominate as sodium, potassium, calcium, and magnesium salts. In a system of only calcium and phosphate ions at the pH of milk, calcium phosphate precipitates; however, the calcium and phosphate as found in milk are in equilibrium with various other ions and the citric acid and casein compete with the phosphate for calcium.

The amount of calcium present in milk in the ionic form is about ten per cent (Table 72) of total calcium, as shown by the murexide method of Smeets.⁵⁹ Using the ion exchange equilibrium method, Christianson *et al.*¹⁰ and van Kreveld and van Minnen³² found

about 8% ionized calcium and 15% ionized magnesium of the total calcium and magnesium, respectively. Although a majority of the calcium is associated with the colloidal fraction, a considerable amount of the calcium remaining in the serum or diffusate is un-ionized and in the form of calcium citrate complexes. Evidently competition between the various acids for the calcium and magnesium ion contribute to the stability of milk.

EQUILIBRIA BETWEEN LIQUID AND COLLOIDAL PHASES

The content of certain salts—notably calcium, magnesium, phosphates, and citrates—in milk is greater than can be maintained in solution. The excess of these salts is present in the colloidal particles associated with the caseinate-phosphate complex. The determination of the distribution of the salts associated with the particles and in solution depends upon the separation of the phases. This has been done by ultrafiltration, high speed centrifugation, equilibrium dialysis, and by coagulation with rennin. The results obtained by the use of these methods for the distribution of the salts between the colloidal particles and the aqueous phase are in general agreement (Table 72). Davies and White also determined the distribution of salts between the dissolved phase and the colloidal phase by ultrafiltration and obtained values for the dissolved salts in essential agreement with those in Table 72. They found that at 3°C. the amount of dissolved salts was greater than at 20°C. by both ultrafiltration and dialysis. This change in soluble salts due to lowering the temperature is reversible and probably is associated with the greater solubility of the β -casein fraction at low temperatures. Because of the change in equilibrium of the salts caused by lowering the temperature, it is desirable to make measurements of the distribution of salts near room temperature. This can be done by dialysis at 20°C. for a period of 48 hr. using a water to milk ratio of 1:50 and 0.25% chloroform as a preservative.

About 56 and 65%, respectively, of the total phosphorus and calcium in milk (Table 72) is associated with the colloidal casein phase and about 42% of this phosphorus is combined with the casein, assuming a nitrogen factor of 6.4 and 0.85% phosphorus for casein. The balance between the salts of the colloidal phase and soluble phase may be upset by various treatments of milk. On the addition of water to milk, the pH is increased and the amount of colloidal casein is reduced.²⁹ However, when milk is concentrated, the pH is reduced and more calcium, phosphate and citrate

accumulate in the colloidal particles¹² even though the ionic strength is increased, which has been found to greatly increase the solubility product of secondary calcium phosphate.⁶⁶ When the pH of skim milk is decreased by the addition of acid, the calcium and phosphate is progressively removed from the casein micelles and at about pH 5.2 the casein precipitates.

The wide variations found for the calcium/casein ratios in the complex in the milk from individual cows⁷⁵ suggest the possibility that equilibrium may shift as a function of the time elapsed after milking due to the loss of carbon dioxide or other changes and also as a result of mixing the milk from two or more cows. Evenhuis and de Vries,¹⁵ however, found no difference in the calcium/casein ratio in the complex when milk from an individual cow was analyzed directly after milking and again after 2, 4, and 6 hr. Also, the results obtained for the calcium/casein ratio of a mixture of milk from two cows, one at the early stage and the other at the late stage of lactation, were the average of the two. It may be concluded from these results that the equilibrium under these conditions is not changed enough to be detected by the methods of analysis.

The equilibrium of ions between the aqueous and colloidal phase can also be determined by means of ion exchange resin. Baker *et al.*³ have measured the rates of removal of calcium and phosphorus from skim milk by resins. The rate constant for the removal of calcium was constant until 90% of the calcium was removed and was also constant for phosphate until 65% of the phosphate was removed. They found two measurable rates of exchange for calcium, a fast one attributed to the ionic and dissolved calcium and a slower one assigned to the exchange with the colloidal phase. Christianson *et al.*¹⁰ found the apparent concentration of ionic calcium and magnesium in milk at 22° and 25°C. to be 2.0 and 2.3 millimoles of calcium and 0.82 and 0.85 millimoles of magnesium. These values are strongly influenced by pH, citrate, and previous heat treatment.

It has been known for a long time that heating milk causes a transfer of calcium and phosphate from solution to the colloidal state. Jenness and Patton²⁹ have summarized the literature showing that the soluble calcium and phosphate are markedly decreased by heating milk at 78°C. for 30 min. but that in about two days standing at room temperature this effect is essentially reversed.

The colloidal phase of milk can be converted to the soluble phase by sequestering agents, of which ethylenediamine tetraacetate, citrate, or oxalate are effective in combining with divalent calcium. When milk is treated with a cation exchanger in the sodium form,

the resin exchanges sodium for calcium and the colloidal phase disappears. Ion exchange resins can effectively substitute potassium for sodium or the milk can be progressively de-ionized by passage through a mixed bed resin.

REFERENCES

- Alexander, T. G., and Ford, T. F., *J. Dairy Sci.*, **40**, 1273 (1957).
- Annibaldi, S., *Lait*, **40**, 593 (1960).
- Baker, J. M., Gehrke, C. W., and Affsprung, H. E., *J. Dairy Sci.*, **37**, 1409 (1954).
- Beeby, R., *J. Dairy Res.*, **30**, 77 (1963).
- Beeby, R., and Kumetat, K., *J. Dairy Res.*, **26**, 248 (1959).
- Bjerrum, J., Schwarzenbach, G., and Sillén, L. G., "Stability Constants," Part I, Chemical Society, London (1957).
- Bohren, H. U., and Wenner, V. R., *J. Dairy Sci.*, **44**, 1213 (1961).
- Burg, P., van der, *Neth. Milk Dairy J.*, **1**, 69 (1947).
- Choate, W. L., Heckman, F. A., and Ford, T. F., *J. Dairy Sci.*, **42**, 761 (1959).
- Christianson, G., Jenness, R., and Coulter S. T., *Anal. Chem.*, **26**, 1923 (1954).
- Davies, D. T., and White, J. C. D., *J. Dairy Res.*, **27**, 171 (1960).
- Eilers, H., Saal, R. N. J., and Waarden, N., van der, "Chemical and Physical Investigations on Dairy Products," p. 20, Elsevier Publishing Co., Inc., Amsterdam (1947).
- Evenhuis, N., and Vries, Th. R., de, *Neth. Milk Dairy J.*, **11**, 111 (1957).
- Evenhuis, N., and Vries, Th. R., de, *Neth. Milk Dairy J.*, **11**, 213 (1957).
- Evenhuis, N., and Vries, Th. R., de, *Neth. Milk Dairy J.*, **13**, 1 (1959).
- Ford, T. F., and Martinez-Mateo, J., *J. Dairy Sci.*, **41**, 1286 (1958).
- Ford, T. F., Ramsdell, G. A., and Alexander, T. G., *J. Dairy Sci.*, **42**, 397 (1959).
- Ford, T. F., Ramsdell, G. A., and Landsman, S. G., *J. Dairy Sci.*, **38**, 843 (1955).
- Ford, T. F., Ramsdell, G. A., Landsman, S. G., and Alexander, T. G., *J. Dairy Sci.*, **40**, 1395 (1957).
- Gordon, W. G., and Semmett, W. F., *J. Am. Chem. Soc.*, **75**, 328 (1953).
- Graham, W. R., Jr., and Kay, H. D., *J. Dairy Res.*, **5**, 54 (1933).
- Groves, M. L., *J. Am. Chem. Soc.*, **82**, 3345 (1960).
- Hetrick, J. H., and Tracy, P. H., *J. Dairy Sci.*, **31**, 867 (1948).
- Heyndrickx, G. V., and Vleeschauwer, A., de, *Experientia*, **8**, 317 (1952).
- Hipp, N. J., Groves, M. L., and McMeekin, T. L., *Arch. Biochem. Biophys.*, **93**, 245 (1961).
- Hostettler, H., and Imhof, K., *Milchwissenschaft*, **6**, 351, 400 (1951).
- Hostettler, H., Rychener, E., and Künzle, L., *Landw. Jahrb. Schweiz*, **62**, 31 (1949).
- Huth, E., *Ann. Paediat.*, **187**, 377 (1956); *Dairy Sci. Abstr.*, **19**, 581 (1957).
- Jenness, R., and Patton, S., "Principles of Dairy Chemistry," p. 332, John Wiley and Sons Inc., New York (1959).
- Kadt, G. S., de, and Minnen, G., van, *Rec. trav. chim.*, **62**, 257 (1943).
- Kirschmeier, O., *Milchwissenschaft*, **17**, 408 (1962).
- Krevelde, A., van, and Minnen, G., van, *Neth. Milk Dairy J.*, **9**, 1 (1955).
- Larson, B. L., Gray, R. S., and Salisbury, G. W., *J. Biol. Chem.*, **211**, 43 (1954).
- Larson, B. L., and Jenness, R., *J. Dairy Sci.*, **38**, 313 (1955).
- Linderstrøm-Lang, K., *Compt. rend. trav. lab. Carlsberg*, **17**, No. 9, 1 (1929).
- Long, J., Van Winkle, Q., and Gould, I. A., *J. Dairy Sci.*, **41**, 317 (1958).
- McGann, T. C. A., and Pyne, G. T., *J. Dairy Res.*, **27**, 403 (1960).
- McKenzie, H. A., and Wake, R. G., *Aust. J. Chem.*, **12**, 734 (1959).
- McMeekin, T. L., and Groves, M. L., unpublished observations (1953).
- McMeekin, T. L., Groves, M. L., and Hipp, N. J., *J. Am. Chem. Soc.*, **71**, 3298 (1949).
- McMeekin, T. L., Hipp, N. J., and Groves, M. L., *Arch. Biochem. Biophys.*, **83**, 35 (1959).
- Manson, W., *16th Int. Dairy Congr. Proc.*, Section IV:1, 513 (1962).
- Manson, W., *J. Electroanal. Chem.*, **3**, 203 (1962).
- Morton, R. K., *Nature*, **171**, 734 (1953).
- Nichols, J. B., Bailey, E. D., Holm, G. E., Greenbank, G. R., and Deysher, E. F., *J. Phys. Chem.*, **35**, 1303 (1931).
- Nielsen, H. C., *Dissertation Abstracts*, **20**, 1152 (1959).
- Nitschmann, Hs., *Helv. Chim. Acta*, **32**, 1258 (1949).
- Pedersen, K. O., *Biochem. J.*, **30**, 948 (1936).
- Polis, B. D., and Shmukler, H. W., *J. Dairy Sci.*, **33**, 619 (1950).
- Pyne, G. T., *J. Dairy Res.*, **29**, 101 (1962).
- Pyne, G. T., and McGann, T. C. A., *J. Dairy Res.*, **27**, 9 (1960).
- Pyne, G. T., and Ryan J. J., *J. Dairy Res.*, **17**, 200 (1950).
- Ramsdell, G. A., and Hufnagel, C. F., *13th Int. Dairy Congress Proc.*, **3**, 1025 The Hague (1953).
- Ramsdell, G. A., and Whittier, E. O., *J. Biol. Chem.*, **154**, 413 (1944).
- Reeves, R. E., and Latour, N. G., *Science*, **128**, 472 (1958).
- Robinson, R. A., and Stokes, R. H., "Electrolyte Solutions," p. 496, Academic Press, New York, (1955).

57. Rose, D., *J. Dairy Sci.*, **44**, 430 (1961).
58. Shimmin, P. D., and Hill, R. D., *J. Dairy Res.*, **31**, 121 (1964).
59. Smeets, W. Th. G. M., *Neth. Milk Dairy J.*, **9**, 249 (1955).
60. Sullivan, R. A., Fitzpatrick, M. M., and Stanton, E. K., *Nature*, **183**, 616 (1959).
61. Sullivan, R. A., Fitzpatrick, M. M., Stanton, E. K., Annino, R., Kissel, G., and Palermi, F., *Arch. Biochem. Biophys.*, **55**, 455 (1955).
62. Svedberg, T., and Chirnoaga, E., *J. Am. Chem. Soc.*, **50**, 1399 (1928).
63. Svedberg, T., and Pedersen, K. O., "The Ultracentrifuge," p. 377, Oxford University Press, London, (1940).
64. Swaisgood, H. E., and Brunner, J. R., *Biochem. Biophys. Res. Comm.*, **12**, 148 (1963).
65. Ter Horst, Maria G., *Neth. Milk and Dairy J.*, **17**, 185 (1963).
66. Tessier, H., and Rose, D., *J. Dairy Sci.*, **41**, 351 (1958).
67. Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, **20**, 135 (1915).
68. Von Hippel, P. H., and Waugh, D. F., *J. Am. Chem. Soc.*, **77**, 4311 (1955).
69. Wake, R. G., and Baldwin, R. L., *Biochim. et Biophys. Acta*, **47**, 225 (1961).
70. Warner, R. C., *J. Am. Chem. Soc.*, **66**, 1725 (1944).
71. Warner, R. C., and Polis, E., *J. Am. Chem. Soc.*, **67**, 529 (1945).
72. Waugh, D. F., *The Faraday Society Discussions*, **25**, 186 (1958).
73. Waugh, D. F., *J. Phys. Chem.*, **65**, 1793 (1961).
74. Waugh, D. F., and Von Hippel, P. H., *J. Am. Chem. Soc.*, **78**, 4576 (1956).
75. White, J. C. D., and Davies, D. T., *J. Dairy Res.*, **25**, 236 (1958).
76. Zittle, C. A., Della Monica, E. S., Custer, J. H., and Rudd, R. K., *J. Dairy Sci.*, **39**, 528 (1956).
77. Zittle, C. A., Della Monica, E. S., Rudd, R. K., and Custer, J. H., *J. Am. Chem. Soc.*, **79**, 4661 (1957).